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(21) International Application Number: PCT/US97/18251 (22) International Filing Date: 3 October 1997 (03.10.97) (30) Priority Data: 60/027,000 4 October 1996 (04.10.96) US (71) Applicant (for all designated States except US): EMBREX, INC. [US/US]; P.O. Box 13989, Research Triangle Park, NC 27709-3989 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): PHELPS, Patricia, V. [US/US]; 8313 Nantahala Drive, Raleigh, NC 27612 (US). (74) Agent: BENNETT, Virginia, C.; Myers Bigel Sibley & Sajovec, LLP, P.O. Box 37428, Raleigh, NC 27627 (US).		(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD OF SORTING BIRDS IN OVO (57) Abstract A method of determining the gender of a bird <i>in ovo</i> comprises detecting the presence or absence of an elevated level of a sex-related hormone in the extra-embryonic fluid of the bird egg, and then determining the gender of the bird within the egg from the presence of an elevated level of a sex-related hormone therein. Preferably, the sex-related hormone is an estrogen. Further preferred are methods in which the extra-embryonic fluid is allantoic fluid. The method is preferably carried out on chicken eggs prior to or during transfer of the eggs from incubator to hatcher.		

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METHOD OF SORTING BIRDS IN OVO

This application claims the benefit of United States Provisional Application No. 60/027,000 filed October 4, 1996.

Field of the Invention

5 The present invention relates to methods of sorting birds by gender, and particularly relates to such methods that can be carried out *in ovo*.

Background of the Invention

10 Commercial birds are generally sorted by gender after hatch. Gender sorting is typically carried out by hand by visual inspection, and can be a time-consuming, tedious, and inaccurate process. Since the failure to properly sort birds by gender can lead to problems in poultry or egg production, it would be extremely useful to have a reliable means for sorting birds by gender without the need for visual inspection of the bird.

15 Accordingly, an object of the present invention is to provide a means for sorting birds by gender.

 A further object of the present invention is to provide a means for sorting birds by gender that can be carried out without the need for visual inspection of the bird.

20 A still further object of the present invention is to provide a means for sorting birds by gender that can be carried out while the birds are *in ovo*.

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Summary of the Invention

The foregoing and other objects and aspects of the present invention may be accomplished by the method of determining the gender of a bird *in ovo* disclosed herein.

As a first aspect, the present invention provides a method of determining the
5 gender of a bird *in ovo*, comprising the steps of: detecting the presence or absence of
an elevated level of a sex-related hormone in the extra-embryonic fluid of a bird egg;
and determining the gender of the bird within the egg from the presence of an elevated
level of the sex-related hormone in the extra-embryonic fluid.

As a second aspect, the present invention provides a method of sorting a
10 plurality of bird eggs by the gender of the birds *in ovo* therein, comprising the steps
of: detecting the presence or absence of an elevated level of a sex-related hormone in
the extra-embryonic fluid of each of the eggs; and then separating the plurality of eggs
into a first subset of eggs having elevated levels of the sex-related hormone and a
second subset of eggs not having elevated levels of the sex-related hormone, so that
15 the first subset of eggs contains birds of one sex and the second subset of eggs
contains birds of the other sex.

As a third aspect, the present invention provides a method of determining
the gender of a bird *in ovo*, comprising the steps of: detecting the presence or absence
of an elevated level of estrogen in the extra-embryonic fluid of a bird egg; and
20 determining that the gender of the bird within the egg is a female from the presence of
an elevated estrogen level in the extra-embryonic fluid.

Yet another aspect of the present invention is a method of sorting a
plurality of bird eggs by the gender of the birds *in ovo* therein, comprising the steps
of: detecting the presence or absence of an elevated estrogen level in the extra-
25 embryonic fluid of each of the eggs; and then separating the plurality of eggs into a
first subset of eggs having elevated estrogen levels and a second subset of eggs not
having elevated estrogen levels, so that the first subset of eggs contains female birds
and the second subset of eggs contains male birds.

These and other aspects of the present invention are described in more
30 detail in the following description of the invention.

Detailed Description of the Invention

Disclosed herein are methods of sorting birds by gender *in ovo* by detecting the presence or absence of an elevated level of a sex-related hormone in the extra-embryonic fluid of the egg. The phrase "sex-related hormone" as used herein, is any hormone that stimulates accessory sex structures and/or secondary sex characteristics in male or female birds. Exemplary sex-related hormones include estrogens and androgens. Alternately, a "sex-related hormone" is a hormone that is present at relatively high concentrations in birds of one gender and are present only at relatively low levels in birds of the other gender. For example, progestogens, which play a role in regulating the estrous cycle and maintaining pregnancy are also encompassed by the term "sex-related hormone". As a further alternative, "sex-related" hormones are those hormones that are characteristic of one of the sexes of birds and, thus, can be used to distinguish one sex from the other. The disclosed methods can be carried out by detecting the levels of more than one sex-related hormone. Generally, however, methods that involve detecting the presence or absence of elevated levels of one sex-related hormone are preferred.

Estrogens include, but are not limited to, estradiol, estradiol 17 β , estriol and estrone. Also included are estrogen precursors, such as dihydroepiandrosterone, metabolic by-products of estrogen degradation, and naturally-occurring estrogen derivatives and variants. Androgens include, but are not limited to, testosterone and dihydrotestosterone. Also included are androgen precursors, such as androstenedione, androstenediol and androstenediol, metabolic by-products of androgen degradation, and naturally-occurring androgen derivatives and variants. Progestogens include, but are not limited to, progesterone and 17-OH progesterone. Also included are progesterone precursors, such as pregnenolone, metabolic by-products of progesterone degradation such as pregnanediol, and naturally-occurring progesterone derivatives and variants.

Preferred are methods of sorting birds by gender *in ovo* which involve detecting the presence or absence of an elevated level of estrogen in the extra-embryonic fluid of the egg. The presence of an elevated level of estrogen in the extra-embryonic fluids is indicative of a female bird. Any estrogen that is secreted into the extra-embryonic fluid in the egg may be used to carry out the present invention, with estradiol being preferred.

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Those skilled in the art will appreciate that the inventive methods disclosed herein also encompass methods of sorting birds by detecting the presence or absence of an elevated level of a sex-related pheromone, protein or enzyme. Such pheromones, proteins or enzymes are present at relatively high concentrations in birds of one sex and at relatively low concentrations in birds of the opposite sex. Further, sex-related pheromones, proteins and enzymes can be used to distinguish one sex from the other.

To describe the present invention in an alternative way, the inventive methods disclosed herein provide a method of determining the sex of a bird *in ovo*, by measuring the level of a sex-related hormone in the extra-embryonic fluid of a bird egg and comparing that level to a predetermined standard, where a measurement above the standard indicates that the bird *in ovo* is of one sex, and a measurement below the standard indicates the bird *in ovo* is of the other sex. The predetermined standard will vary depending upon the species of bird being examined, the age of the egg at the time of examination, the acceptable percentage of error, and the hormone being examined.

Experiments to determine a standard useful in a particular setting may be carried out using methods available in the art, and would be apparent to one skilled in the art (*see, e.g.,* Examples 1-6 herein). In a preferred method, the eggs are chicken eggs, the hormone is estrogen, and the extra-embryonic fluid is allantoic fluid.

The terms "bird" and "avian" as used herein, are intended to include males or females of any avian species, but are primarily intended to encompass poultry which are commercially raised for eggs or meat. Accordingly, the terms "bird" and "avian" are particularly intended to encompass hens, cocks and drakes of chickens, turkeys, ducks, geese, quail and pheasant. Chickens and turkeys are preferred, with chickens being most preferred.

The term "*in ovo*," as used herein, refers to birds contained within an egg prior to hatch. The present invention may be practiced with any type of bird egg, including chicken, turkey, duck, goose, quail, and pheasant eggs. Chicken and turkey eggs are preferred, with chicken eggs most preferred.

Eggs sorted by the method of the present invention are fertile eggs which are preferably in the last half of incubation, more preferably in the third quarter of incubation. The detecting or sorting step may be carried out on chicken eggs on about the eleventh to nineteenth day of incubation, more preferably on about the thirteenth to

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seventeenth day of incubation, and most preferably on about the thirteenth to fifteenth day of incubation. The detecting step is preferably carried out on turkey eggs on about the fourteenth to twenty-sixth day of incubation, more preferably on about the sixteenth to twenty-first day of incubation, most preferably on about the sixteenth to nineteenth day of incubation. Those skilled in the art will appreciate that the present invention can be carried out at any predetermined time *in ovo*, as long as the level of a sex-related hormone in the extra-embryonic fluid at the chosen time is a reliable predictor of the sex of the bird.

According to the methods of sorting birds disclosed herein, the level of one or more sex-related hormones in the extra-embryonic fluid in the egg are measured, the level of the sex-related hormone being indicative of the sex of the bird. The extra-embryonic fluid can be from any source in the egg, including but not limited to allantoic fluid, amniotic fluid, yolk fluid, albumen fluid, or blood. There is no requirement that the fluid come from a single source – it may be advantageous to combine two or more of these fluids, *e.g.*, to obtain sufficient volume on which to carry out analyses. Preferably, the extra-embryonic fluid is allantoic fluid.

While the methods of the present invention may be carried out on individual eggs, in a commercial setting the method is typically carried out on a plurality of eggs. In general, in a commercial setting, a plurality of eggs are incubated together in a common incubator. At about the beginning of the final quarter of incubation, the eggs are transferred from the incubator to a hatcher. This step is known as "transfer". The step of detecting the presence or absence of an elevated level of a sex-related hormone in the extra-embryonic fluid of each egg may be carried out prior to or after the transfer step. The detecting step can also be carried out at the time of transfer. Preferably, the detecting step is carried out prior to or at the time of the transfer of eggs from the incubator to the hatcher. Some methods of detecting the level of a sex-related hormone will cause a time delay while the diagnostic assays are being performed. According to this embodiment of the invention, after the hormone analyses are complete, it is preferable to sort and transfer the eggs to the hatcher at the same time.

The detecting step may be carried out by any suitable means which can detect differences in the levels of a sex-related hormone. The detection step may involve noninvasive techniques such as infrared spectroscopy analysis through the egg's shell or

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inner shell membranes or by inserting a biosensor into the appropriate target area (*e.g.*, the allantoic sac if the target fluid is allantoic fluid), or by inserting a needle therein and withdrawing a fluid sample therefrom, which sample is subsequently subjected to an analysis such as by radioimmunoassay, infrared spectroscopy, photoacoustics, artificial nose techniques, or immunosensor techniques.

An "elevated" level of a sex-related hormone is a level that is at or above the normal range for an egg of the desired sex. For example, an "elevated" level of estrogen is a level that is at or above the normal range for an egg containing a female bird. Alternately, an elevated level of a sex-related hormone is a level that is sufficiently high to distinguish one gender of bird from the other *in ovo*, *i.e.*, a level of estrogen that is sufficiently high to distinguish female from male birds. As a further alternative, an "elevated" level of sex-related hormone is a level at or above a predetermined level, such that the predetermined level can be used as a reliable predictor of sex in a *bird in ovo*. An "elevated" level of estrogen may be considered an estrogen level of 20, 40, 60, 80, or 100 picograms per milliliter or more in the target fluid. Likewise, an "elevated" level of a sex-related hormone may be considered a level of 20, 40, 60, 80, 100, or 200 picograms per milliliter or more in the target fluid.

After the detecting step, the plurality of eggs is then separated into a first subset of eggs having an elevated level of the sex-related hormone of interest, and a second subset of eggs not having elevated levels of the same sex-related hormone, so that said birds are sorted by gender. For example, if the indicator hormone is an estrogen, the first subset of eggs contains female birds and the second subset of eggs contains male birds. Where there is a time delay between withdrawing a sample from the eggs and obtaining the results of the diagnostic test, the eggs can simply be marked with a label, wax pencil, or other indicia or other suitable marking means that corresponds to an indicia associated with the sample withdrawn, and the eggs subsequently sorted when the results from the diagnostic tests are available.

After separating the eggs into a first and second subset, the eggs are separately incubated to hatch. This step may be conveniently carried out by simply placing the eggs in separate hatchers.

The present invention is explained in greater detail in the following non-limiting examples.

EXAMPLES 1-6

5 Allantoic and amniotic fluids were sampled from either Day 11 or Day 17 chicken embryos. The Day 17 embryos were then necropsied and the sex determined by visual observation of the gonads. Blood samples were collected from the Day 11 embryos, frozen and set aside for PCR analysis for the W specific chromosome to determine embryonic sex. The samples were then subjected to a radioimmunoassay (Coat-A-Count Estradiol kit, Diagnostic Products Corporation) to determine the amount of estradiol therein. The results are presented in Table 1.

TABLE 1

Embryo Age	Fluid	Sex	Estradiol (pg/ml)
17	allantois	F	260 414 266
17	allantois	M	UD UD 15
17	amnion	F	4 17 11
17	amnion	M	UD 9 UD
11	allantois	TBD	24 69 UD 12 UD UD UD 27
11	amnion	TBD	UD UD UD UD UD UD

UD = Undetectable

TBD= To Be Determined

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A method of determining the gender of a bird *in ovo*, comprising the steps of:

detecting the presence or absence of an elevated level of a sex-related hormone in the extra-embryonic fluid of a bird egg; and

5 determining the gender of the bird within the egg from the presence of an elevated level of said sex-related hormone in said extra-embryonic fluid.

2. A method according to claim 1, wherein said extra-embryonic fluid is allantoic fluid.

3. A method according to claim 1, wherein said detecting step is carried out by a method selected from the group consisting of radioimmunoassay, infrared spectroscopy, artificial nose analysis, photoacoustics, and immunosensor analysis.

4. A method according to claim 1, wherein said egg is a chicken egg.

5. A method according to claim 1, wherein said detecting step is carried out during the last half of incubation.

6. A method according to claim 4, wherein said detecting step is carried out from day 11 to day 19 of incubation.

7. A method according to claim 4, wherein said detecting step is carried out from day 13 to day 17 of incubation.

8. A method of sorting a plurality of bird eggs by the gender of the birds *in ovo* therein, comprising the steps of:

detecting the presence or absence of an elevated level of a sex-related hormone in the extra-embryonic fluid of each of said eggs; and then

5 separating said plurality of eggs into a first subset of eggs having elevated

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levels of said sex-related hormone and a second subset of eggs not having elevated levels of said sex-related hormone, so that said first subset of eggs contains birds of one sex and said second subset of eggs contains birds of the other sex.

9. A method according to claim 8, further comprising the step of incubating said eggs together in a common incubator prior to said detecting step.

10. A method according to claim 8, wherein said separating step is followed by the step of separately incubating said first subset of eggs and said second subset of eggs to hatch.

11. A method according to claim 8, further comprising the step of transferring said birds to a hatcher after said detecting step.

12. A method according to claim 8, further comprising the step of transferring said birds to a hatcher concurrently with said detecting step.

13. A method according to claim 8, wherein said egg is a chicken egg.

14. A method according to claim 8, wherein said detecting step is carried out during the last half of incubation.

15. A method according to claim 13, wherein said detecting step is carried out from day 11 to day 19 of incubation.

16. A method according to claim 13, wherein said detecting step is carried out from day 13 to day 17 of incubation.

17. A method according to claim 8, wherein said extra-embryonic fluid is allantoic fluid.

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18. A method according to claim 8, wherein said detecting step is carried out by a method selected from the group consisting of radioimmunoassay, infrared spectroscopy, artificial nose analysis, photoacoustics, and immunosensor analysis.

19. A method of determining the gender of a bird *in ovo*, comprising the steps of:

detecting the presence or absence of an elevated level of estrogen in the extra-embryonic fluid of a bird egg; and

5 determining that the gender of the bird within the egg is a female from the presence of an elevated estrogen level in said extra-embryonic fluid.

20. A method according to claim 19, wherein said extra-embryonic fluid is allantoic fluid.

21. A method according to claim 19, wherein said detecting step is carried out by a method selected from the group consisting of radioimmunoassay, infrared spectroscopy, artificial nose analysis, photoacoustics, and immunosensor analysis.

22. A method according to claim 19, wherein said egg is a chicken egg.

23. A method according to claim 19, wherein said detecting step is carried out during the last half of incubation.

24. A method according to claim 22, wherein said detecting step is carried out from day 11 to day 19 of incubation.

25. A method according to claim 22, wherein said detecting step is carried out from day 13 to day 17 of incubation.

26. A method of sorting a plurality of bird eggs by the gender of the birds *in ovo* therein, comprising the steps of:

detecting the presence or absence of an elevated estrogen level in the extra-embryonic fluid of each of said eggs; and then

5 separating said plurality of eggs into a first subset of eggs having elevated estrogen levels and a second subset of eggs not having elevated estrogen levels, so that said first subset of eggs contains female birds and said second subset of eggs contains male birds.

27. A method according to claim 26, further comprising the step of incubating said eggs together in a common incubator prior to said detecting step.

28. A method according to claim 26, wherein said separating step is followed by the step of separately incubating said first subset of eggs and said second subset of eggs to hatch.

29. A method according to claim 26, further comprising the step of transferring said birds to a hatcher after said detecting step.

30. A method according to claim 26, further comprising the step of transferring said birds to a hatcher concurrently with said detecting step.

31. A method according to claim 26, wherein said egg is a chicken egg.

32. A method according to claim 26, wherein said detecting step is carried out during the last half of incubation.

33. A method according to claim 31, wherein said detecting step is carried out from day 11 to day 19 of incubation.

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34. A method according to claim 31, wherein said detecting step is carried out from day 13 to day 17 of incubation.

35. A method according to claim 26, wherein said extra-embryonic fluid is allantoic fluid.

36. A method according to claim 26, wherein said detecting step is carried out by a method selected from the group consisting of radioimmunoassay, infrared spectroscopy, artificial nose analysis, photoacoustics, and immunosensor analysis.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/74 A01K67/00

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WENIGER J.P.: "Embryonic Sex Hormones in Birds" INT. J. DEV. BIOL., 1991, pages 1-8, XP002056536 see the whole document ---	1-36
X	WENIGER J.P.: "Recherches sur la Nature Chimique des Hormones Sexuelles Embryonnaires de Poulot" ANN. EMBRYOL. MORPHOG., vol. 2, 1969, pages 433-444, XP002056537 see the whole document ---	1-36
A	EP 0 433 084 A (MERCK & CO INC) 19 June 1991 see abstract ---	1

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☒ Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

information on patent family members

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